Specific Analysis in Plasma and Urine of 2,3-Dinor-5,6-dihydro-isoprostane $F_{2\alpha}$-III, a Metabolite of Isoprostane $F_{2\alpha}$-III and an Oxidation Product of γ-Linolenic Acid*

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F_2-isoprostanes (iPs) are free radical-catalyzed isomers of prostaglandin $F_{2\alpha}$. Circulating and urinary iPs have been used as indices of lipid peroxidation *in vivo*. Utilizing an ^{18}O-labeled homologous internal standard, we developed a gas chromatography/mass spectrometry assay for the 2,3-dinor-5,6-dihydro (dinor-dihydro) metabolite of $iPF_{2\alpha}$-III. Although urinary excretion of $iPF_{2\alpha}$-III reflects systemic lipid peroxidation, the metabolite is more abundant (median of 877 (range of 351–1831) *versus* 174 (range of 56–321) pg/mg of creatinine; $p < 0.01$) than the parent iP in urine and can be measured in plasma. Metabolite analysis may be preferable in plasma due to the abundance of arachidonic acid as a source of *ex vivo* lipid peroxidation. Also, $iPF_{2\alpha}$-III may be formed in blood samples in a cyclooxygenase-dependent manner by platelets *ex vivo*. By contrast, the metabolite is not formed by aggregated platelets (0.71 ± 0.08 *versus* 0.65 ± 0.09 pg/ml). Although the metabolite/parent ratio is altered in cirrhosis, urinary dinor-dihydro-$iPF_{2\alpha}$-III is elevated and increases further during reperfusion following orthotopic liver transplantation. In addition to its formation as an iPF_2 metabolite, analysis of γ-linolenic acid autooxidation products and the compound present in freeze-thawed plasma suggests that γ-linolenic acid may also be an important source of dinor-dihydro-$iPF_{2\alpha}$-III.

Isoprostanes (iPs)[1] are free radical-catalyzed products of arachidonic acid (1, 2). F_2-iPs, isomers of prostaglandin $F_{2\alpha}$, are the most studied species, but analogous isomers (isoeicosanoids) of other prostaglandins as well as of leukotrienes have been described (3, 4). Isoeicosanoids are formed initially *in situ* in the phospholipid domain of cell membranes by a free radical attack on polyunsaturated fatty acids. They are subsequently cleared by phospholipases, enter the general circulation, and are excreted in urine. Given their relative chemical stability,

iPs represent attractive indices of oxidant stress *in vivo*. Indeed, iPs have been measured in human atherosclerotic plaque and localized to monocytes/macrophages and vascular smooth muscle cells (5). Urinary excretion of discrete F_2-iPs is increased in humans and mice with hypercholesterolemia (6, 7). Suppression of urinary iP excretion with vitamin E in apoE-deficient mice retards atherogenesis, despite sustained hypercholesterolemia (7).

Despite the emerging evidence supporting the quantitation of iPs as an approach to the study of oxidant stress *in vivo* (8, 9), little is known about their release from cell membranes or their metabolic disposition prior to excretion. Several analytical approaches to quantitation of F_2-iPs have been employed (2). Those based on GC/MS have usually measured a heterogeneous GC peak composed of an undetermined number of isomers. We have developed assays that measure a single isomer, which may be more reproducible. To date, we have developed methods for $iPF_{2\alpha}$-III (formerly 8-iso-prostaglandin $F_{2\alpha}$) (10), $iPF_{2\alpha}$-VI (formerly $iPF_{2\alpha}$-I) (11), and 8,12-iso-$iPF_{2\alpha}$-VI (12). We have reported coordinate elevation of distinct iPs in the atherosclerotic plaque (5) in the brains of patients with Alzheimer's disease (13) as well as in the urine of cigarette smokers (14), patients with hypercholesterolemia (6), and patients with chronic obstructive pulmonary disease (15). Immunoassays for $iPF_{2\alpha}$-III have been developed (16, 17), and some have been commercialized. However, the cross-reactivity of these antibodies with most other F_2-iPs and their putative metabolites is unknown. Administration of labeled $iPF_{2\alpha}$-III to primates and one human suggested that close to 20% of administered radioactivity recovered in urine is 2,3-dinor-5,6-dihydroisoprostane $F_{2\alpha}$-III (dinor-dihydro-$iPF_{2\alpha}$-III) (18). More recently, Chiabrando *et al.* (19) identified the presence of 2,3-dinor-$iPF_{2\alpha}$-III, in addition to the 2,3-dinor-5,6-dihydro metabolite, in human urine. Although this assay did not include use of homologous internal standards, the authors estimated the quantities of both metabolites in human urine as being roughly equivalent to the parent compound, $iPF_{2\alpha}$-III.

There is a substantial rationale for the development of sensitive and specific assays for iP metabolites. Thus, we have previously reported that $iPF_{2\alpha}$-III, unlike other iPs, may be formed by COX isozymes *in vitro* or *ex vivo* (10, 20). Although this pathway of formation contributes to an undetectable extent to urinary $iPF_{2\alpha}$-III excretion, even under conditions of COX activation (14), it may confound quantitation of iPs in blood samples (11, 16). Similarly, peroxidation of arachidonate substrate may represent an additional source of *ex vivo* artifact in blood sampling, although this problem may be circumvented by analysis in urine, where there is considerably less substrate (6, 11). Measurement of a metabolite, if not formed in the cells of circulating blood, may bypass these sources of *ex vivo* artifact

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[1] The abbreviations used are: iPs, isoprostanes; GC/MS, gas chromatography/mass spectrometry; dinor-dihydro-$iPF_{2\alpha}$-III, 2,3-dinor-5,6-dihydro-isoprostane $F_{2\alpha}$-III; COX, cyclooxygenase; GLA, γ-linolenic acid; SPE, solid-phase extraction; PRP, platelet-rich plasma.

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(21). Morrow *et al.* (22) have recently reported a GC/MS assay for dinor-dihydro-iPF$_{2\alpha}$-III using an $^{18}O_2$-labeled homologous internal standard. We have independently developed a similar assay and report elevated levels in patients with hepatic cirrhosis. Urinary dinor-dihydro-iPF$_{2\alpha}$-III is further increased during the reperfusion phase following hepatic transplantation. Unlike its parent compound, iPF$_{2\alpha}$-III, this metabolite is not formed in plasma by activated platelets. However, dinor-dihydro-iPF$_{2\alpha}$-III may also be formed as a primary iP from the autoxidation of an alternative fatty acid substrate, γ-linolenic acid (GLA). Although GLA may be a less prevalent substrate than arachidonic acid in human tissues, the similarity of the array of isomers observed following autoxidation of GLA and those observed in human urine and plasma implies that this may be a significant source of dinor-dihydro-iPF$_{2\alpha}$-III. This confounding duality suggests that interpretation of dinor-dihydro-iPF$_{2\alpha}$-III levels is less than straightforward. However, either as a primary iP derived from GLA or as a metabolite of an arachidonic acid-derived iP, urinary dinor-dihydro-iPF$_{2\alpha}$-III, just like iPF$_{2\alpha}$-III, reflects alterations in lipid peroxidation *in vivo*, even in patients with hepatic dysfunction.

EXPERIMENTAL PROCEDURES

Analysis in Urine—Synthetic dinor-dihydro-iPF$_{2\alpha}$-III was generously provided by Cayman Chemical Co., Inc. (Ann Arbor, MI). An internal standard of [$^{18}O_2$]dinor-dihydro-iPF$_{2\alpha}$-III was formed following the method of Pickett and Murphy (23). Unless otherwise specified, all organic solvents were of analytical grade (Burdick & Jackson, Muskegon, MI). Each 1-ml sample of urine was spiked with 1.5 ng of internal standard and acidified with formic acid to pH <3. The metabolite was extracted by solid-phase extraction (SPE) on a RapidTrace SPE workstation (Zymark Corp., Hopkinton, MA) using C18 EC cartridges (100 mg of sorbent; International Sorbent Technology, Mid Glamorgan, United Kingdom). The solvent program included conditioning the cartridge with 3 ml of ethanol followed by 0.5 ml of pH 3 buffer. The sample was loaded onto the cartridge, rinsed with 2 ml of pH 3 buffer followed by 1 ml of 12.5% ethanol and 0.5% acetic acid in water, and eluted with 1 ml of ethyl acetate. After SPE, the sample was then dried under N_2 and redissolved in 30 μl of methanol. The sample was purified by TLC on silica gel plates (LK6D, Whatman) using a mobile phase of 20% methanol, 80% ethyl acetate, and 0.1% acetic acid. Two micrograms of iPF$_{2\alpha}$-III was applied to a separate plate to visualize the zone to be scraped. The plate was then dipped in 8% H_3PO_4 in 0.3 M $CuSO_4$ and heated on a hot plate. An 8-mm zone was scraped from the sample plate at 0.94 times the R_F of the standard. The pentafluorobenzyl ester was formed using 10 μl of diisopropylethylamine (Sigma) and 20 μl of 1% pentafluorobenzyl bromide (Sigma) in acetonitrile and allowing the sample to stand at room temperature for 10 min. The samples were dried under N_2, redissolved in 30 μl of ethyl acetate, and further purified by a second TLC step using a mobile phase of 100% ethyl acetate. The zone scraped was 0.82 times the R_F of the iPF$_{2\alpha}$-III pentafluorobenzyl ester standard. Prior to GC/MS, the trimethylsilyl ether derivative was formed by allowing the sample to sit at room temperature for 10 min in 10 μl each of dry pyridine (Mallinckrodt Baker, Paris, KY) and bis(trimethylsilyl)trifluoroacetamide (Supelco Inc., Bellefonte, PA). The reagents were dried under N_2, and the sample was redissolved in 20 μl of dodecane (Sigma) for analysis. A Hewlett-Packard 5973 mass spectrometer interfaced with a Hewlett-Packard 6890 gas chromatograph and a Hewlett-Packard 7683 autosampler was used for quantitation. The gas chromatograph was programmed from 190 to 260 °C at 30 °C/min. We used a 30 m × 0.25 mm, 0.25-μm coating, DB5-MS column (J & W Scientific, Folsom, CA). The carrier gas was helium at an average linear velocity of 30 cm/s. The retention time was ~8 min. The ions monitored were at *m/z* 543.4 and 547.4. Peak area ratios were used for quantitation. Urinary creatinine levels were determined by a spectrophotometric technique (24), enabling dinor-dihydro-iPF$_{2\alpha}$-III levels to be expressed as pg/mg of creatinine.

Freeze-Thaw Treatment of Plasma—One-hundred milliliters of blood was drawn from a healthy subject into tubes containing sodium citrate and centrifuged at 1050 × *g* for 10 min at 4 °C to prepare plasma, 40 ml of which was frozen at −20 °C and then placed at 4 °C for 8 h/day for 14 days. It was then extracted by the method of Bligh and Dyer (25). The resulting emulsion was frozen and recentrifuged to assist in separation of the phases. The lower phase was dried under a stream of nitrogen,

TABLE I
Clinical details of orthotopic liver transplant patients

Age in years	Gender	Origin of their disease
59	M	Hepatitis C
51	M	Hepatitis C
24	F	Wilson's disease
67	M	Primary sclerosing cholangitis
66	F	Hepatitis C + B
58	M	Cryptogenic cirrhosis
47	M	Hepatitis C + alcohol
20	M	Fulminant hepatic failure
39	M	Hepatitis C + alcohol

dissolved in 1 ml of 7.5% KOH in 50% MeOH/H_2O, and heated to 60 °C for 1 h. The pH was then lowered to ~3.0 with 1 N HCl, and the solution was extracted with 3 volumes of EtOAc and dried under N_2. The resulting solution was stored in 1 ml of CH_2Cl_2 at −20 °C until analysis.

Autoxidation of GLA—γ-Linolenic acid (10 mg) and benzoyl peroxide (12.5 mg) were dissolved in 1 ml of CH_2Cl_2 and allowed to stand at room temperature for 72 h. Triphenylphosphine (5 mg) was added, and after standing for 30 min, the solution was dried under a stream of nitrogen. The reaction mixture was stored in 1 ml of acetonitrile at −20 °C until analysis.

Design of Clinical Studies—Two clinical studies were performed. The first assessed the capacity of human platelets to generate the metabolite. The second addressed its formation in patients with liver disease. In the platelet study, 20 ml of blood was drawn from the antecubital veins of five volunteers (three males) aged 27–56 years (mean of 35.2 years). Blood was centrifuged at 200 × *g* for 15 min at room temperature, and 2.4 ml of the platelet-rich plasma (PRP) was removed. The remainder of the sample was centrifuged at 1000 × *g* at 4 °C for 10 min. A 2.4-ml aliquot of this platelet-poor plasma supernatant was removed and stored on ice until used. Fifteen microliters of collagen (Chronolog Corp., Havertown, PA) was added to each of the PRP samples, which were stirred at 37 °C for 5 min to induce platelet aggregation and then placed on ice. Two-milliliter aliquots of each sample were transferred to a clean test tube, and plasma protein was denatured and precipitated by the addition of 2 ml of ice-cold 10% isopropyl alcohol in acetonitrile. The samples were then supplemented with 91.75 pg/ml [$^{18}O_2$]dinor-dihydro-iPF$_{2\alpha}$-III and 400 pg of [$^{18}O_2$] iPF$_{2\alpha}$-III, acidified, and centrifuged. The supernatant was transferred to a glass test tube for automated solid-phase extraction. C18 cartridges were conditioned with 1 ml of ethanol followed by 0.3 ml of pH 3 buffer. The sample was loaded and rinsed with 1 ml of pH 3 buffer and 1 ml of 25% ethanol in water with 0.1% acetic acid. The column was dried with 10 ml of air, and the sample was collected in 1 ml of ethyl acetate. This was purified by straight-phase SPE using Isolute NH$_3$ cartridges (International Sorbent Technology). The sample was loaded, rinsed with 50% acetonitrile in ethanol, and collected in 5% acetic acid in methanol. This was dried, and the pentafluorobenzyl derivative was formed. TLC purification was performed as described for urine samples. iPF$_{2\alpha}$-III was separated from the metabolite by scraping at the level of the visualization standard after the second TLC step, and a *tert*-butyldimethylsilyl ether derivative was formed as described previously (10). The ions monitored by GC/MS were at *m/z* 547.4 and 543.4 for the dinor-dihydro compound and at *m/z* 699.5 and 695.5 for iPF$_{2\alpha}$-III.

Healthy volunteers (*n* = 32) used as controls in the liver study were aged 20–57 years (15 males). Patients with liver disease, including some scheduled to undergo orthoptic liver transplantation, were recruited from the Hepatology Clinic at the University of Pennsylvania Medical Center. Their clinical details are provided in Table I. All patients who participated in the study consented to do so, or if unable to give consent, this was obtained on their behalf from family members. Base-line urine samples were obtained prior to orthoptic liver transplantation. Intraoperative samples were obtained prior to the start of the anhepatic phase of surgery, prior to reperfusion, 15–30 and 90–120 min after reperfusion of the newly grafted liver. Samples were immediately placed on ice and then stored at −80 °C until analysis.

RESULTS

Sensitivity and Specificity of the Assay—Dinor-dihydro-iPF$_{2\alpha}$-III was easily identifiable in urine and clearly separated from other compounds of the same mass (Fig. 1). The intra-assay variability was 3.35%, and the interassay variability was 4.47% on a single sample assayed six times on 4 separate days

m/z 547

m/z 543

Time (min)

FIG. 1. GC/MS of dinor-dihydro-iPF$_{2\alpha}$-III in urine. The *upper trace* (*m/z* 547) represents [^{18}O$_2$]dinor-dihydro-iPF$_{2\alpha}$-III, and the *lower trace* (*m/z* 543) represents endogenous dinor-dihydro-iPF$_{2\alpha}$-III. *PFB*, pentafluorobenzyl; *TMS*, trimethylsilyl.
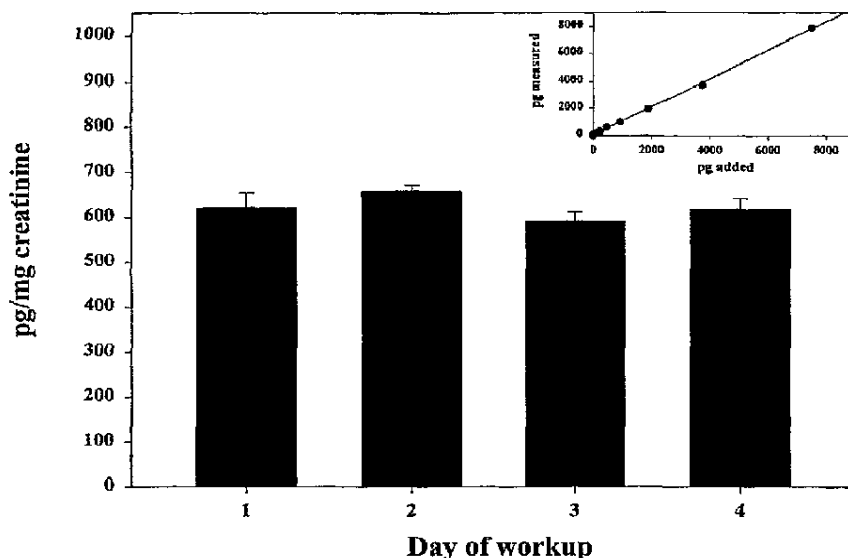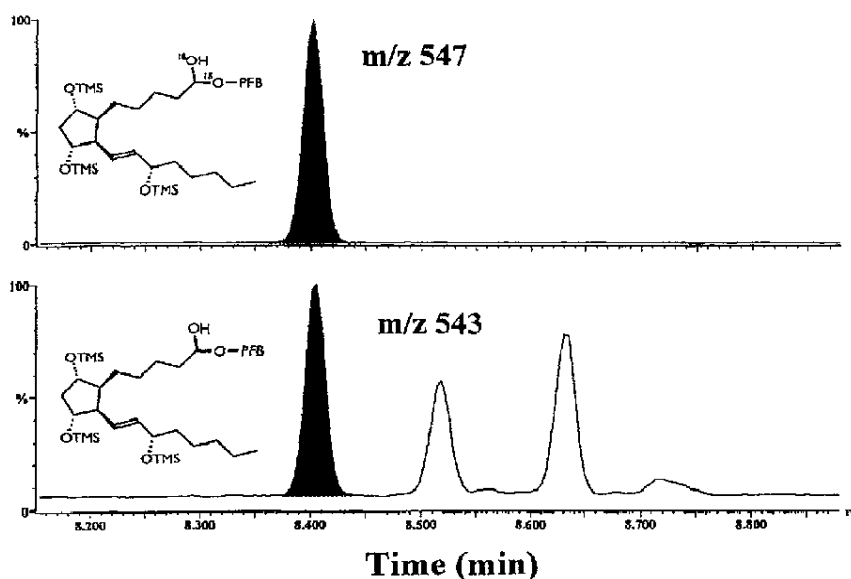

Day of workup

FIG. 2. Reproducibility and linearity of the GC/MS assay for dinor-dihydro-iPF$_{2\alpha}$-III. A urine sample was worked up six times on each of 4 days. The *inset* shows the linearity of the assay over the range of 0–8000 pg of authentic dinor-dihydro-iPF$_{2\alpha}$-III added per ml.

(Fig. 2). The assay was linear over a range of 0–8000 pg of added authentic material (Fig. 2). Twenty-four-hour urine samples collected from 32 healthy volunteers gave a mean urinary dinor-dihydro-iPF$_{2\alpha}$-III level of 540.7 ± 61 (range of 132–1434) pg/mg of creatinine. Values in males (541 ± 90.5 pg/mg of creatinine, aged 22–57 years) were not significantly different from those in females (540.4 ± 85.5 pg/mg of creatinine, aged 20–56 years). There was no significant relationship (r^2 = 0.008) between urinary dinor-dihydro-iPF$_{2\alpha}$-III and age in the total group. Paired analysis of both compounds was performed in a subset of 12 healthy individuals. Urinary levels of the dinor-dihydro metabolite were significantly higher (median of 876.9 (range of 351–1831) pg/mg of creatinine) than those of iPF$_{2\alpha}$-III (median of 174 (range of 56–321) pg/mg of creatinine; p < 0.01) in these individuals.

iPF$_{2\alpha}$-III and Its Dinor-dihydro Metabolite in Urine—Both urinary iPF$_{2\alpha}$-III and dinor-dihydro-iPF$_{2\alpha}$-III were analyzed in

a subset of 12 healthy volunteers and 11 patients with chronic liver disease. Urinary iPF$_{2\alpha}$-III was elevated in liver disease, as we have previously reported (26, 27). The urinary dinor-dihydro metabolite was also elevated (3750 ± 847.4 *versus* 876.9 ± 148 pg/mg of creatinine) in patients with chronic liver disease compared with controls. The ratio of the dinor-dihydro compound to parent compound, iPF$_{2\alpha}$-III, was 5.3 ± 8.5 in healthy controls (n = 12) and 3.3 ± 0.56 in age- and gender-matched patients (n = 22) with chronic liver disease. The difference between these ratios attained statistical significance (p < 0.005).

The dinor-dihydro compound is not formed by activated platelets. Levels of the parent isoprostane rose from 2.4 ± 0.62 pg/ml in platelet-poor plasma to 29.2 ± 7.5 pg/ml in aggregated PRP (p < 0.0001). By contrast, levels of the metabolite were unchanged (0.71 ± 0.08 *versus* 0.65 ± 0.09 pg/ml; p = not significant) (Fig. 3).

Elevated Metabolite Excretion during Hepatic Reperfusion—
Ten patients due to undergo orthoptic liver transplantation were studied. Eight were male. Preoperative urinary metabolite levels in these patients with chronic liver disease were also significantly ($p < 0.01$) elevated (1914.5 ± 416.6 pg/mg of creatinine). Serial intraoperative samples were available from seven of these patients. Urinary dinor-dihydro-iPF$_{2\alpha}$-III increased further ($p < 0.01$) during the reperfusion phase after hepatic ischemia, peaking at 3213.7 ± 686.4 pg/mg of creatinine 90–120 min post-reperfusion. Excretion of the metabolite decreased over the next several days to the range observed in healthy volunteers (Fig. 4).

*Formation as a Primary Isoprostane from GLA—*Autoxidation of GLA *in vitro* gave rise to a series of peaks, the largest of which coeluted with the internal standard for dinor-dihydro-iPF$_{2\alpha}$-III (Fig. 5). The pattern of peaks observed in plasma closely resembles that of autooxidized GLA. Furthermore, the large increase in all peaks after plasma is subjected to freeze-thaw treatment is likely analogous to the observation by Morrow *et al.* (28) of similar increases in the concentration of arachidonic acid-derived F$_2$-iPs after such treatment. The fact that a similar pattern of peaks was observed in normal urine (Fig. 5) further implies that a significant portion of urinary dinor-dihydro-iPF$_{2\alpha}$-III is also derived from autoxidized GLA. A schematic route for the formation of GLA-derived iPs is depicted in Fig. 6.

DISCUSSION

We report the presence of dinor-dihydro-iPF$_{2\alpha}$-III in human urine and plasma using a highly reproducible, specific, and sensitive GC/MS-based assay. Previously, Roberts *et al.* (18) reported that intravenous administration of labeled iPF$_{2\alpha}$-III to non-human primates and to a human volunteer resulted in ~20% of the radioactivity in urine being accounted for by this compound. Thus, it is likely that endogenous iPF$_{2\alpha}$-III is a source of endogenous urinary dinor-dihydro-iPF$_{2\alpha}$-III in the present study. Chiabrando *et al.* (19) have identified the presence of both dinor-dihydro-iPF$_{2\alpha}$-III and 2,3-dinor-iPF$_{2\alpha}$-III in human urine. They demonstrated that excretion of the metabolites was elevated in smokers and was not depressed in individuals given the COX inhibitor naproxen. Their approach to quantitation is accompanied by several caveats, however. Due to the lack of homologous stable isotope-labeled internal standards, they could not control for the efficiency of the immunoaffinity extraction. Also, the possibility exists for differential efficiency of MS fragmentation between the [^{2}H$_4$]iPF$_{2\alpha}$-III in-
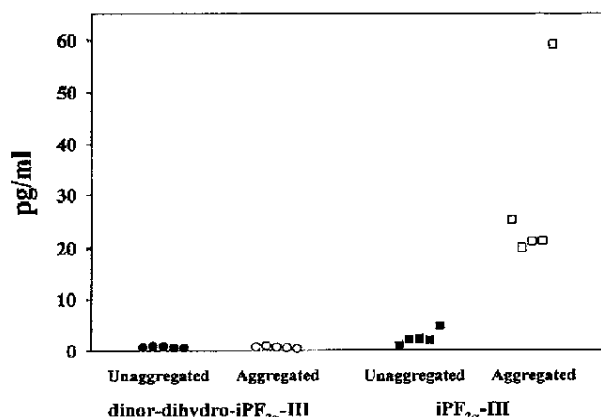
FIG. 3. Effect of platelet aggregation on dinor-dihydro-iPF$_{2\alpha}$-III and iPF$_{2\alpha}$-III. PRP and platelet-poor plasma were prepared as described under "Experimental Procedures." PRP was aggregated with collagen, and all samples were then extracted, purified, derivatized, and analyzed by GC/MS as described under "Experimental Procedures."
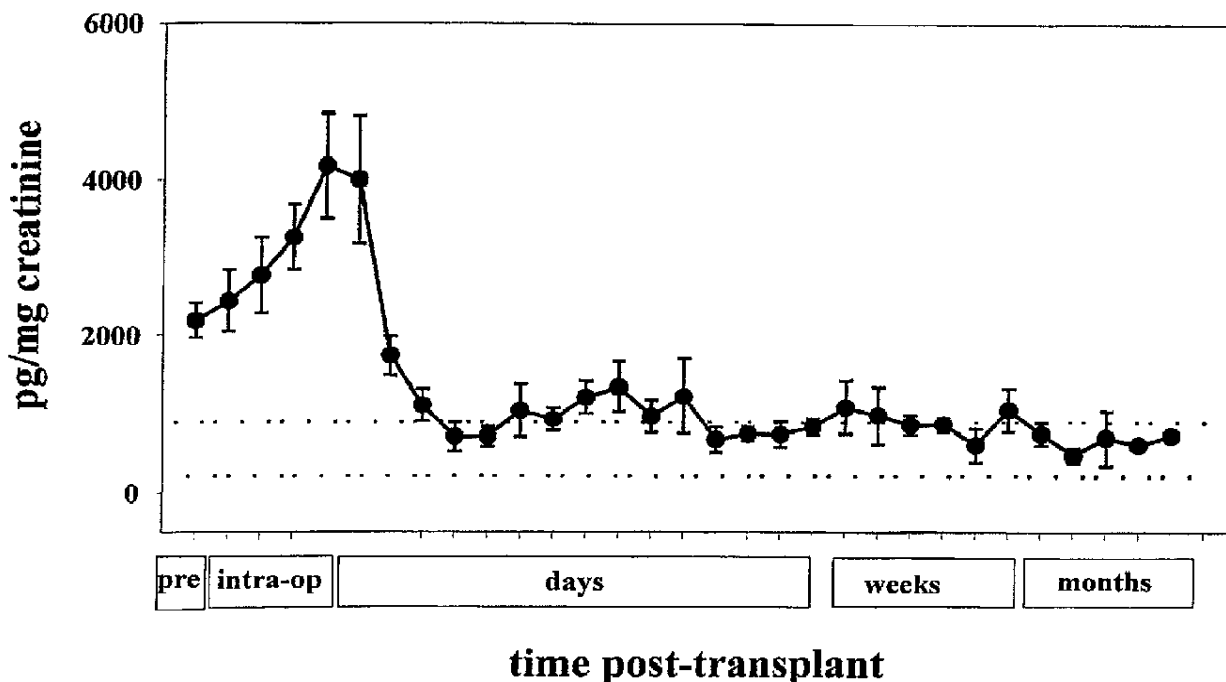
time post-transplant

FIG. 4. Urinary dinor-dihydro-iPF$_{2\alpha}$-III excretion before and after liver transplantation. Levels were elevated pre-transplant, increased further with reperfusion of the grafted liver, and returned to the level of healthy volunteers (*dotted lines*) within 4 days of transplant. *intra-op*, intraoperative.
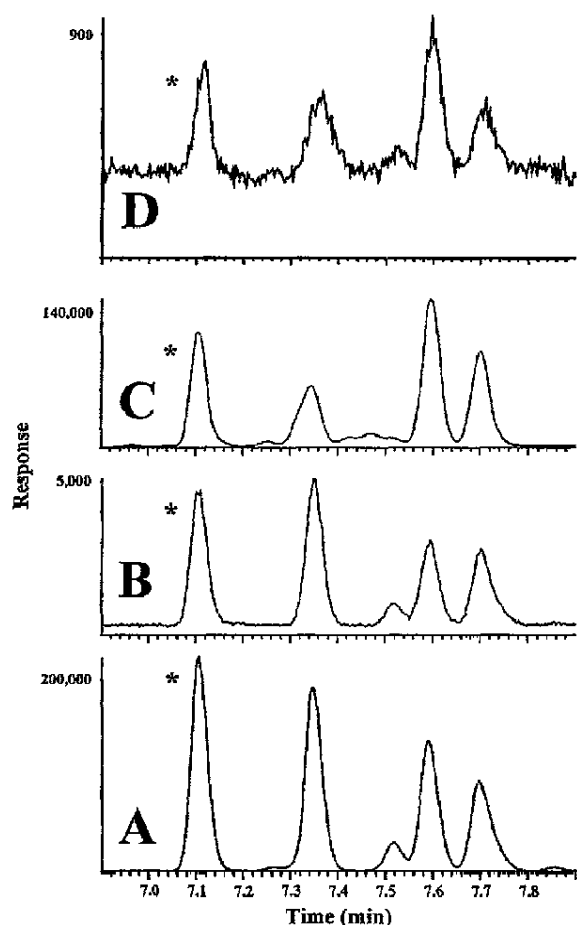
FIG. 5. GC/MS analysis after purification and derivatization. All traces represent selected ion monitoring at m/z 543. A, autoxidized GLA; B, normal human urine; C, freeze-thawed human plasma; D, normal human plasma. Samples were either supplemented before workup (B and D) or co-injected (A and C) with synthetic $[^{18}O_2]$dinor-dihydro-iPF$_{2\alpha}$-III. For clarity, the selected ion monitoring trace for the standard (m/z 547), which coeluted with the peaks marked with *asterisks*, is not shown.

ternal standard and the two metabolites.

Urinary dinor-dihydro-iPF$_{2\alpha}$-III, just like iPF$_{2\alpha}$-III (26, 27, 29, 30), is increased in patients with hepatic cirrhosis, as are other markers of oxidant stress (26, 30). In the present study, there was an additional transient increment in metabolite excretion during the reperfusion phase following orthoptic liver transplantation. We have previously described such an increment in urinary iPF$_{2\alpha}$-III coincident with coronary reperfusion after ischemia with thrombolytic drugs, cardiopulmonary bypass (31), or angioplasty (32). Interestingly, in the present study, urinary excretion of the dinor-dihydro compound fell to levels within the normal range after reperfusion, suggesting correction of the preoperative syndrome of oxidant stress. We are presently exploring the possibility that later increases in metabolite excretion might presage episodes of transplant rejection.

Although measurement of parent iPs in urine reflects systemic and not just renal generation of iPs (26, 27, 31, 32), analysis of a metabolite rather than the parent iP may be advantageous in certain circumstances. We have previously demonstrated that iPF$_{2\alpha}$-III, apparently alone among the F$_2$-iPs, can be formed in a COX-dependent manner by platelets *in*

*vitro* and in serum *ex vivo* (10, 16). Both COX-1 in platelets (10) and COX-2 in monocytes (20) have the capacity to form iPF$_{2\alpha}$-III. Although this enzymatic pathway does not appear to contribute detectably to urinary concentrations of the iP (11), even in syndromes of COX activation (14), this may not be the case for plasma-based assays. Thus, COX-1 activation in platelets will occur *ex vivo* after blood sampling and may confound estimates of iPF$_{2\alpha}$-III actually circulating *in vivo*. For example, thromboxane A$_2$, the major COX metabolite formed by platelets (33), is rapidly hydrolyzed to thromboxane B$_2$, which is biologically inactive, but chemically relatively stable and readily measured in biological fluids. However, continuing platelet activation *ex vivo*, even when samples are harvested into a mixture including a COX inhibitor, results in an uncontrollable source of artifact *ex vivo* (21). In the case of thromboxane, this is readily addressed by measurement of a metabolite (11-dehydro thromboxane B$_2$) that is not formed by cells of the circulating blood (21), but rather in tissues such as liver, lung, and kidney. We now report that the dinor-dihydro metabolite of iPF$_{2\alpha}$-III, unlike the parent isoprostane, is not formed by aggregated human platelets.

In the case of the iPs, a second form of potential artifact is lipid peroxidation *ex vivo*. This may also be more of a problem for plasma-based assays of parent iPs, where the content of the arachidonic acid substrate is much greater than in urine. Again, this problem may be bypassed by measurement of a metabolite that is not formed in blood. Formation of parent iPs *ex vivo* in urine has not been detected in carefully collected samples (11).

In addition to these theoretical advantages of a metabolite assay in plasma, such compounds are sometimes more abundant than the parent compound in urine. In contrast to the findings of Chiabrando *et al.* (19), we report that this is also the case for dinor-dihydro-iPF$_{2\alpha}$-III. The greater abundance of the metabolite renders it a more tractable target for various forms of analysis. In this case, the GC/MS method we describe for the metabolite is more facile than that for the parent compound, iPF$_{2\alpha}$-III (10). Use of the trimethylsilyl derivative allows for a reaction time of 10 min as opposed to 12 h for the *tert*-butyldimethylsilyl, a GC retention time of 8 *versus* 13 min, and a smaller sample volume of urine (1 *versus* 5 ml). Although lacking the elegant simplicity possible with immunoaffinity extraction, this method takes advantage of reasonably priced, commercially available, single-use SPE cartridges and TLC plates, avoiding the necessity of raising, purifying, characterizing, and coupling antibodies (19).

The availability of assays for iP metabolites will permit initial studies of iP disposition. Although we do not know the tissues in which this or other metabolites are formed, the liver seems one likely source by analogy with dinor metabolites of prostaglandins and thromboxane. Indeed, the dinor-dihydro metabolite is formed from iPF$_{2\alpha}$-III by rat hepatocytes *in vitro* (19). Consistent with the likelihood of a hepatic contribution to metabolite biosynthesis in humans, the ratio of metabolite to parent in the urine of patients with cirrhosis is less than that observed in healthy individuals. However, even in liver disease, urinary levels of both the metabolite and the parent iP are elevated compared with controls, suggesting that a change in iP generation predominates over one in iP disposition. Paired analysis of parent plus metabolite will permit further elucidation of alterations in generation *versus* disposition of iPs in human disease. Biological effects have been attributed to iPs, including iPF$_{2\alpha}$-III (2). The extent to which metabolic disposition contributes to the inactivation of such effects of parent iPs *in vivo* is presently unknown. Recently, Basu (34) described an additional compound, 2,3,4,5-tetranor-15-keto-13,14-dihydro-

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FIG. 6. **Free radical-catalyzed autooxidation of GLA.** Two classes of F_1-isoprostanes, each composed of eight isomers, are theoretically possible. For further details of this process, see Ref. 37. *FR*, free radicals.
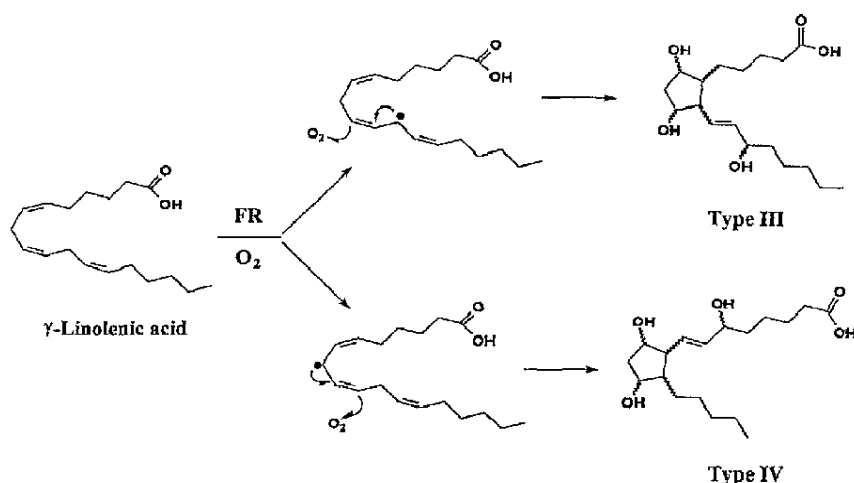
$iPF_{2\alpha}$-III, as a major metabolite in the rat.

Little is known of the effects of substrate modification on iP generation. Peroxidation products of both eicosapentaenoic acid and docosahexaenoic acid have been described (35). Interestingly, aside from its formation as a metabolite of arachidonic acid-derived iP ($iPF_{2\alpha}$-III), the dinor-dihydro compound may theoretically also be formed as a primary iP from GLA, which itself may be formed as a degradation product of arachidonic acid. Interestingly, $iPF_{2\alpha}$-III is degraded to dinor-dihydro-$iPF_{2\alpha}$-III by the same peroxisomal mechanism that converts arachidonic acid to GLA (Fig. 6). Dinor isoprostane regioisomers formed from α-linolenic acid in plants have previously been described (36). We present evidence consistent with the identity of dinor-dihydro-$iPF_{2\alpha}$-III in human urine and plasma to a primary autoxidation product of GLA. We cannot discriminate between the two potential sources of the dinor-dihydro compound in the present study. However, the pattern of peaks observed in urine and plasma bears a striking similarity to the pattern obtained from the autoxidation of GLA, suggesting that this may be a significant source of the compound. Indeed, a more appealing nomenclature for the compound would be 2,3-dinor-$iPF_{1\alpha}$-III (37), which is unencumbered by the implication of a metabolic derivation from $iPF_{2\alpha}$-III.

In summary, we describe the detection of dinor-dihydro-$iPF_{2\alpha}$-III (2,3-dinor-$iPF_{1\alpha}$-III) in human plasma and urine using a homologous standard in a GC/MS assay. Urinary excretion of the compound was elevated in patients with cirrhosis and was increased further during the reperfusion phase of hepatic transplantation. Irrespective of whether it is formed as a metabolite of arachidonic acid-derived iP or directly from GLA, it is not formed by activated platelets and is more abundant and more readily measured than $iPF_{2\alpha}$-III in urine; and urinary levels reflect lipid peroxidation *in vivo*.

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